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TEN PRINCIPLES OF HETEROCHROMATIN FORMATION AND FUNCTION

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Abstract

Heterochromatin is a critical architectural unit of eukaryotic chromosomes. It endows particular genomic domains with specific functional properties. Critical is the role of heterochromatin in genomic stability, which is mediated by its ability to restrain mobile elements, isolate repair events in repetitive regions, and to contribute to the formation of structures that ensure accurate chromosome segregation. This distinctive chromatin also contributes to developmental regulation by restricting the accessible compartment of the genome in specific lineages. The establishment and maintenance mechanisms that mediate heterochromatin assembly are separable and involve the ability of sequence-specific factors, modified chromatin and nascent transcript-bound proteins to recruit chromatin-modifying enzymes. Heterochromatin can spread along the chromatin fiber from nucleation sites and also mediates its own epigenetic inheritance through cell division, yet these propensities are normally strongly repressed. Due to its central importance in chromosome biology, heterochromatin plays key roles in the pathogenesis of various human diseases. In this article, we derive these broadly conserved principles of heterochromatin formation and function using selected examples from studies of a range of eukaryotic model organisms from yeast to man, with an emphasis on insights obtained from unicellular systems.

Introduction

Heterochromatin is a fundamental architectural unit of eukaryotic chromosomes that endows particular genomic regions with specific functional properties. The term “heterochromatin” was coined based on the differential staining of chromosomal regions, but now generally refers to molecular subtypes of repressed domains that extend beyond a single gene or regulatory element [Box 1]. Different varieties of heterochromatin are distinguished by their combination of modifications of histone side chains. These impact the recruitment of proteins as well chromatin fiber folding. Sequences embedded in heterochromatin often contain repetitive elements, such as satellite repeats, transposable elements, and transposon remnants. A critical function of heterochromatic packaging is to prevent such selfish nucleic acids and tandem repeats from producing genetic instability. Heterochromatin exhibits additional roles, including functions in cell type-specific transcription and centromere function.

Histones are subject to post-translational modifications (PTMs), particularly on lysine residues within the unstructured tails protruding from nucleosomes. Such modifications are often referred to as ‘epigenetic marks’ as they can confer properties to a chromosomal region not strictly dependent on DNA sequences in that region [Box 2]. Histone PTMs regulate the propensity of the underlying DNA to participate in the processes of transcription, replication, repair and recombination. Specific PTMs control binding of particular proteins to the nucleosome via specific domains (**Fig. 1**). Such ‘reader’ modules can be joined to enzyme domains that modify chromatin, or are part of complexes that contain or recruit such enzymes. Enzymes modules that modify histones are termed ‘writers’; those that remove modifications ‘erasers.’ Another type of enzyme recruited by histone modifications are chromatin remodelers that alter contacts between the histone octamer core and DNA to accomplish a variety of tasks[1].

The best-studied types of heterochromatin are marked by the addition one (Mono; me1), two (Di; me2) or three (Tri; me3) methyl groups to lysine 9 or 27 of the H3 tail (H3K9me, H3K27me). Here we focus mostly on H3K9me-dependent heterochromatin, which forms the major blocks of heterochromatin in cells, and represents the defining molecular feature of constitutive heterochromatin in many eukaryotes (**FIG. 1a**). We also touch on other paradigms to highlight specific concepts. Histone H3K9 methylation is catalyzed ("written") by SET domains of orthologs of the Drosophila Suppressor-of-variegation-3-9 (SU(VAR)3-9; referred to as SUV39H1 and SUV39H2 in mammals and more generally abbreviated here as Suv39), and fission yeast (*Schizosaccharomyces pombe*) Cryptic-loci-regulator-4 (Clr4) lysine methyltransferases (KMTs; **Fig. 1b**)[2-4]; this modification recruits HP1 family "reader" proteins (Drosophila: Heterochromatin Protein 1a and 1b; *S. pombe*: Switch-6, Swi6 and Chromo-domain-protein-2, Chp2) which harbor chromo-domains that selectively bind the H3K9 methyl mark[2-13]. While H3K9 methylation and its recognition are important, additional molecular players and histone modifications contribute. Moreover, in some systems, repressive histone methylation can be coupled to DNA methylation (position 5 on cytosine; 5meC)[14-17].

Because the information content of the field has exploded in recent years, our goal here is to derive the key principles of heterochromatin formation and function. We illustrate these with examples taken mainly from unicellular yeasts, but include selected studies from a variety of model organisms. It is not our intention to be comprehensive, and so we have limited discussion of system-specific details and caveats. Below we discuss studies and approaches that illuminate how histone modifications recruit heterochromatin components, the role of RNA as a recruiting platform, the differences between heterochromatin establishment and maintenance, the processes of heterochromatin spreading and inheritance, and the contributions of heterochromatin to genome defense, development and disease.

1. Reader-modifier coupling

While distinct from canonical heterochromatin marked by H3K9me, the Silent-Information-Regulator (SIR) system of the budding yeast, *Saccharomyces cerevisiae* (reviewed in [18,19]) was the first system in which players involved in the silencing of a chromosome domain and their mechanisms of action were molecularly defined. In this species, silencer elements are recognized by sequence-specific DNA binding proteins that then recruit four proteins: Sir1, Sir2, Sir3 and Sir4. Sir2 is an NAD-dependent histone deacetylase (HDAC) which acts on acetylated lysine 16 of histone H4 (H4K16ac), enabling the Bromo-associated homology (BAH) domain of Sir3 (a component of the Sir3-Sir4 complex) to bind nucleosomes[20]. Sir2 deacetylation thus promotes Sir3 binding, allowing further cycles of Sir protein recruitment to form silent domains. The SIR system illustrates the principle of reader-modifier coupling (**FIG. 1c**), in this case between the Sir3 reader and the Sir2 eraser. It also illustrates the principle of initial recruitment by sequence-specific DNA binding proteins (**FIG. 1d**). While paradigmatic, the SIR system is a relatively recent evolutionary invention, restricted to *S. cerevisiae* and its relatives[21].

Nonetheless, reader-modifier coupling is also a key feature of the more canonical H3K9me-marked heterochromatin[5,6,22-24]. Both *Drosophila* and mammalian Suv39 and *S. pombe* Clr4 H3K9 methylases have a similar layout with an N-terminal chromo-domain and C-terminal SET domain (**FIG. 1b**), coupling writer and reader modules in the same polypeptide. Methylation of H3K9 by the SET domain enables recruitment of Suv39 or Clr4 via their respective chromo-domains. HP1 proteins contain not only a chromo-domain reader module, but also a more C-terminal chromoshadow domain (CSD; **FIG. 1a).** CSD dimerization forms a binding platform for other effector proteins[25,26]. Reading of the H3K9me mark by HP1 proteins thus enables another route to reader-modifier coupling through the CSD dimer platform. For example, recruitment of HDAC (eraser) complexes (SHREC; Snf2/HDAC-Repressor-Complex and Clr6-Complex) by Swi6 and Chp2 removes acetylation allowing nearby H3K9 methylation in *S. pombe*[27,28]. The recruitment of SHREC, which harbors the

Mit1 (Mi2-like-interacting) remodeler subunit, also plays a role in the elimination of nucleosome-free regions, whose absence is a hallmark of heterochromatin in *S. pombe*[29,30]. Being part of dimeric proteins, the reader domains of HP1 proteins are also coupled as pairs with ensuing functional consequences: two dimers of Swi6 bind a single H3K9me-modified nucleosome, providing "sticky ends" that enable Swi6 to bridge two nucleosomes[31]. In some systems, H3K9 readers can be coupled to DNA modification. In mammals and plants, 5meC DNA methyltransferases are recruited in tandem with H3K9 methyltransferases bolstering each other to ensure that the encompassed DNA is rendered inaccessible[15-17].

Reader-modifer coupling is also a feature of the more dynamic silencing complexes recruited by H3K27me. Methylation of H3K27 by Enhancer-of-zeste KMTs (Ez, Drosophila; Ezh2, homolog 2, mammals: subunit of Polycomb Repressive Complex 2; PRC2) promotes binding of the Polycomb protein to chromatin (component of Polycomb Repressive Complex 1; PRC1) via its chromo-domain[32-36]. In addition, the methyltransferase complex harbors a subunit (Extra-sex-combs, ESC, Drosophila; Embryonic-ectoderm-development, Eed, mammals) that recognizes the H3K27me mark and allosterically activates Ez and Ezh2 catalytic activities[37] .

2. ncRNAs recruit chromatin modifiers

Heterochromatic regions are transcribed to non-coding RNAs that may be processed to small RNAs[16,38]. This may seem surprising since heterochromatin induces transcriptional silencing. Nonetheless, a low level of transcription occurs in heterochromatin and this is important for heterochromatin formation in several systems. Heterochromatin transcription can be cell cycle regulated, occurring during replication when heterochromatin becomes accessible[39-41]. One function for this transcription appears to be silencing factor recruitment (**FIG. 1d**) via nascent transcripts association, as exemplified by work in *S. pombe* [42-50]. The transcript also provides a substrate for small RNA generation, these small RNAs promote silencing factor recruitment via base-pairing, likely with nascent transcripts.

In *S. pombe* RNA polymerase II (RNAPII) transcribes heterochromatin repeats. The Argonaute protein, Ago1, uses bound single-stranded small interfering siRNAs to target homologous nascent repeat transcripts emerging from chromatin-associated RNAPII and recruits silencing factors[47,51,52]. Ago1 is part of a three-subunit complex, RNA-Induced Transcriptional Silencing (RITS)[46], which associates with both the RNA-dependent RNA polymerase complex (RDRC)[53] and the histone H3K9-KMT Clr4 methyltransferase complex (CLRC) [45,54-56]. RDRC appears to use primary transcripts to template dsRNA biosynthesis for subsequent processing to siRNAs, thereby amplifying siRNA production[53,57]. CLRC is required for all H3K9me while H3K9me promotes efficient siRNA production; thus a positive feedback loop is engaged. Such feedback is, in part, mediated through recruitment of RITS, which contains a chromo-domain protein (Chp1) subunit that binds H3K9me[24,58,59]. Two bridging factors connect the effector complexes: Stc1 (Signals-to-chromatin) recruits CLRC via RITS[44], while Ers1 (Essential-for-RNAi-dependent-silencing) couples RDRC, RITS, and Swi6[HP1 60-62].

In plants, template transcription also provides a feedback loop that promotes H3K9me. Most details come from studies of *Arabidopsis thaliana*. Similar to *S. pombe*, nascent transcripts provide the platform for Argonaute-siRNA complex recruitment. However, RNAPV, a specialized RNAPII paralog, produces those transcripts that are targeted by siRNA-guided AGO4[63]. AGO4 recruits the *de novo* DNA methylase DRM2 (domains-rearranged-methylase)[64], which in turn recruits adaptor KMTs and H3K9 KMTs (SUVH4, SUVH6 and SUVH9) via the DDR complex (Defective-in-RNA-directed-DNA-Methylation-1 - DRD1; Defective-in-Meristem-Silencing-3 - DMS3; RNA-Directed-DNA-Methylation-1 - RDM1)[65]. DNA methylation and H3K9me are also linked through the SRA (SET-and-Ring-finger-associated) domain of KMTs which bind methylated DNA. dsRNAs are produced by another RNAPII paralog called RNAPIV in association with RDRP, their processing by Dicer generates siRNAs that are loaded into AGO4[66-68]. At many sites, RNAPIV is recruited by an

H3K9me reader protein, Shh1 (SAWADEE-homeodomain-homolog), closing another feedback loop[69,70]. Thus, as in *S. pombe*, nascent transcripts have two functions: recruiting chromatin-modifying enzymes using siRNA-transcript base-pairing (via RNAPIV transcription) and templating siRNA production (via RNAPV transcription).

Another role for nascent heterochromatin transcripts is to recruit silencing-promoting proteins without the intermediary of small RNAs. *S. pombe* possesses an RNAi-independent pathway that promotes H3K9me and functions to maintain pericentromeric heterochromatin[71]. One component of this pathway is Seb1 (Seven-binding), a nascent transcript-binding protein which contains an RNA recognition motif (RRM, RNA-binding domain) that recognizes GUA trinucleotides and the RNAPII C-terminal-domain interaction domain[72,73]. Seb1 acts upstream of the SHREC complex [74], which participates in an RNAi-independent pathway[75]. The Seb1-SHREC pathway is partially redundant with RNAi since only in double mutants, where both pathways are inactivated, is H3K9me eliminated[75]. As GUA trinucleotides occur frequently, how Seb1 selectively promotes H3K9me at pericentromeric regions is not known, but GUA sequences are depleted from *S. pombe* protein-coding genes[73]. Moreover, recent findings show that Suv39 KMTs are stabilized on heterochromatin by their non-specific affinity for nascent RNA emanating from mammalian centromere repeat arrays [76-78].

Similar transcription-driven processes mediate X chromosome inactivation in female mammals, a process that produces a condensed, silenced chromosome, marked by H3K27me3. Although the inactive X is not “constitutive heterochromatin”, this form of silent chromatin serves to illustrate related important principles. ˇThe “A-repeat” region of the long non-coding RNA X-inactive specific transcript (XIST) recruits SPEN^{SHARP} (Split-ends), a protein that contains RRM type RNA-binding domains[79-82]. SPEN^{SHARP} in turn recruits HDAC3 histone deacetylase via the SMRT (silencing-mediator-for-retinoid-and thyroid-hormone-receptors) adaptor protein[31,83,84]. Ensuing histone deacetylation probably triggers

the recruitment of at least two redundant repression mechanisms, one being the Polycomb system, while the other remains to be identified (reviewed in [84]). As with Seb1, it is unclear if SPEN^{SHARP} alone has sufficient specificity to target XIST-RNA and the X chromosome for inactivation.

3. Establishment is separable from maintenance

Some signals and factors required to initiate *de novo* heterochromatin assembly (that is to convert euchromatin to heterochromatin) differ from those required for its maintenance. This distinction between establishment and maintenance phases is critical for understanding how heterochromatin formation occurs.

Testing whether a non-essential factor is required to establish heterochromatin (an "establishment factor") is performed in *S. pombe* as follows (**FIG. 2**). Heterochromatin is first erased by removing the gene encoding the key modifier (example: KMT or HDAC). The re-introduction of that gene into otherwise wild-type cells allows heterochromatin re-establishment; however, cells lacking an establishment factor are unable to assemble heterochromatin[24]. Another approach compares the outcome of introducing naïve DNA templates (example: centromere repeats) into wild-type versus mutant cells[24,85,86]. A third way is to erase heterochromatin by exposure to inhibitors (example: HDAC inhibitor Trichostatin A) and determine if mutant cells recover heterochromatin after inhibitor removal[87,88]. Such assays revealed that RNAi plays an essential role in establishing heterochromatin. For instance, in the absence of RNAi factors, no H3K9me can be targeted to centromere repeats or related sequences when Clr4-KMT is re-introduced into cells lacking Clr4. Likewise, H3K9me is established on repeats transformed into wild-type but not cells lacking RNAi. This stands in contrast to the partially redundant role of RNAi (with Seb1, or the HDACs SHREC/Clr3 or Sir2) in the maintenance of H3K9me at pericentromeric regions, in which double mutants between RNAi and Seb1 or an HDAC is required to eliminate H3K9me2 [75,86].

Establishment of heterochromatin on *S. pombe* centromeric outer repeats requires RNAi but it remains unclear how the initiating source of dsRNA is generated. Possibilities include dsRNA produced by convergent, overlapping transcripts[38], secondary structures[89] and degradation products[90]. Another possibility is that the RDRC synthesizes the initiating dsRNA from centromere repeat transcripts[53,57], as in plants (see above). In the latter case, specific features must distinguish repeat element transcripts from mRNA-producing transcripts to specifically recruit RDRC.

In *S. pombe*, dsRNA, induced by expression of an artificial hairpin-encoding DNA, is sufficient to generate synthetic siRNAs and direct H3K9me heterochromatin formation in *cis* at the locus producing dsRNA[91]. Here no inherent special features are required to trigger heterochromatin formation once dsRNA is synthesized. Surprisingly, siRNA produced from such artificial dsRNAs only weakly induce heterochromatin assembly in *trans* at transcribed homologous euchromatic loci[92]. Such synthetic siRNA sources trigger more efficient H3K9me heterochromatin formation in *trans* in cells harboring mutations in the RNAPII-associated Polymerase-associated-factor (PAF) complex[93-95]. Defective canonical polyadenylation signals at the transcribed target locus also enhance silencing[96]. Thus, nascent transcripts may be held at native heterochromatin loci due to inefficient transcriptional elongation/termination, bolstering RNAi-mediated H3K9me formation.

RNAi-independent establishment mechanisms also exist in *S. pombe* since RNAi is not required for establishment of heterochromatin adjacent to telomeres. Clr4-KMT is recruited to telomere repeats via the Shelterin complex, using its telomere repeat DNA binding subunits[97]. However, RNAi contributes to subtelomeric silencing in *S. pombe* via centromere-related telomere-adjacent repeats[98,99].

Separable establishment factors for H3K9 methylation have also been identified in *C. elegans.* In the germline, small Piwi-associated RNAs (piRNAs) trigger an siRNA-H3K9me feedback loop, much like those of *S. pombe* and plants[100]. Once piRNAs have acted, however, they are dispensable for the maintenance of that feedback loop. This was revealed through genetic crosses that removed the two Piwi-related genes, *prg-1* and *prg-2,* after triggering heterochromatin formation[101]. Piwi also plays a role in in the establishment of HP1a-marked heterochromatin during Drosophila development[102].

In *Arabidopsis*, where DNA methylation and H3K9me are linked, most loci controlled by RNAi display the ability to re-establish silencing following transient disruption of the defined feedback loops[103]. However, at a small subset of these loci DNA methylation cannot be rescued by the re-introduction of maintenance DNA methytransferase MET1 to *MET1* mutants[103]. This suggests that once DNA methylation has been erased from these particular loci, they lack the required cues for its re-establishment.

Finally, during X chromosome inactivation in murine epiblasts, the XIST ncRNA gene was shown to be required to establish silencing on one homolog (see above). However, conditional removal of XIST later in development demonstrated that it is not required for maintenance of silencing[104,105]. Analyses in ES cell models shows that SPEN^{SHARP} (and other factors) are required to establish XIST-mediated gene silencing following induced XIST expression[79-82]. The subsequent installation of DNA methylation over the inactive X ensures the inheritance of silencing without XIST or associated factors.

4. Heterochromatin can spread

Once nucleated at a particular location, the biochemical properties of heterochromatin components enable domain expansion that is largely independent of the DNA sequences encountered. The classic example of this is Drosophila PEV where chromosome

translocations juxtapose heterochromatin with euchromatin (reviewed in [106]). In such cases, heterochromatin spreads over large distances into euchromatin. In Drosophila additional heterochromatin titrates limiting factors away from, and consequently weakens, heterochromatin thereby alleviating repression at other locations[107-109]. Thus, spreading requires a supply of surplus, unassembled heterochromatin components, and can be driven by their over-expression [110-112].

Spreading requires reader-writer coupling. Nucleosomes bearing H3K9me are bound by H3K9me writers (Suv39, Clr4) via their chromo domains. Mutants in the Clr4 chromodomain impede spreading in *S. pombe*[45,113]. However, spreading also requires the HP1-dependent recruitment of HDAC activity[28,50,53,114]. Thus, interconnections between reader, writer and eraser modules results in critical positive feedback loops, mediated by reader-modifier coupling, that extend heterochromatin domains.

Single cell reporter analysis in *S. pombe* show that de novo nucleation of the heterochromatin domain at the mating type locus can take several cell divisions while expansion of the domain to its full size needs even longer[115]. Such results predict that feedback mechanisms acts both locally, on adjacent nucleosomes, and more broadly over greater distances to mediate this two step process[115]. Thus, the spreading of silent chromatin does not necessarily occur in a linear fashion; random collisions between a heterochromatin domain and chromatin that is spatially located nearby may allow the key modification to be deposited discontinuously in 'hops' that decline in frequency with distance from the nucleation site or domain. Subsequently, gaps between the original domain and the new patch could then be filled by a pincer-like movement, although exceptions to this scenario have been observed in Drosophila[109]. Modelling of available data suggests that read-write driven feedback, coupled to collisions between modified and unmodified sites, may optimally describe the dynamics of heterochromatin domains[116].

Such models may be impacted by recent findings that describe a role for HP1-induced phase-separation in heterochromatin assembly[117,118]. Purified Drosophila HP1a can form proteinaceous liquid droplets that phase-separate *in vitro* (liquid-liquid demixing) under particular conditions[118]. In Drosophila cells, heterochromatin domains display properties characteristic of phase-separated liquids[118]. *In vitro* demixing has also been reported for the human HP1α protein. Phosphorylated HP1α demixes more efficiently than unphosphorylated HP1α, suggesting potential for regulation *in vivo*[117]. Indeed, a mutant that cannot be phosphorylated forms smaller heterochromatic foci when introduced into cells. Nucleosomes and DNA preferentially partition into these phosphor-HP1α droplets *in vitro*, suggesting that the HP1α 'solvent' may control entry of molecules into heterochromatin[117]. We anticipate that future work will reveal further the function(s) of phase-separation in heterochromatin assembly and/or function.

Mammalian X chromosome inactivation is initiated by XIST expression from the X inactivation centre (XIC). XIST spreads discontinuously over the X chromosome and may first affect non-contiguous chromosomal regions that contact its XIC in three-dimensional space. XIST spreading, accompanied by gene silencing, is not limited to X chromosomes[119,120]. Rearrangements which fuse autosomes to an inactive X result in spreading of silencing into the autosome, albeit with limited efficiency[121-123]. Likewise, ectopic expression of XIST from autosomes results in reduced expression over large adjacent domains[84,104,124-128].

5. Heterochromatin spread is restrained

Because heterochromatin can spread, mechanisms to restrict its expansion are necessary to avoid erroneous, and potentially deleterious, gene silencing (**Figure 3**). Mechanisms described to create such barriers and interrupt lateral heterochromatin spreading include: 1.

nucleosome depleted regions generated by bound proteins such as transcription factors; 2. processes that promote nucleosome turnover; 3. anti-silencing activity recruitment by ongoing transcription and associated regulatory elements; 4. anti-silencing factors recruitment by heterochromatin itself; 5. restricting silencing factors to their sites of prior action.

tRNA genes are a conserved class of boundary. They have been shown to restrict heterochromatin spread in organisms from yeast to man[129-131]. Binding sites for the RNAPIII transcription factor TFIIIC appear to be critical as clusters of these sites alone, independent of tRNA genes, function as boundaries. One example derives from the boundaries of the silent mating type region in *S. pombe*[132]. These regions display large nucleosome-free regions, which may prevent spreading by forming a 'gap' in the chromatin fiber over which some read-write mechanisms cannot cross[29] (**FIG. 1b**). tRNA genes, like the TFIIIC sites at the mating type locus, are themselves accessible and essentially nucleosome-free[133-135]. Turnover of nucleosomes assembled in heterochromatin is low[93,136], and factors such as RNAPII-associated Paf1C, which promote their turnover, are required for boundary function[93,95,136] (**FIG. 1c**). Myraid other boundary element types and factors have been described suggesting that there are likely to be many mechanisms for interrupting heterochromatin assembly.

Euchromatin is marked by a variety of chromatin modifications that antagonize heterochromatin assembly. These include the histone variant H2AZ which is deposited in response to nucleosome free regions in the first nucleosome (+1) downstream of transcription initiation sites[137-139] and histone PTMs triggered by active transcription (acetylation and methylation at specific lysines). It is well recognized that such PTMs (H3K4me, H3K36me and H3K79me) play an anti-silencing role in *S. cerevisiae*, which utilizes SIR-mediated heterochromatin (see above)[140-145]. Thus, transcription induces histone PTMs that restrict heterochromatin formation (**FIG. 3d**). Because heterochromatin inhibits

transcription, transcription instigates a positive-feedback mechanism that stabilizes the euchromatic state and antagonizes the heterochromatic state. Likewise, positive feedback is a feature of robust heterochromatin assembly. Competition between these two opposing, positive feedback mechanisms likely explain the bi-stability of alternative chromatin states inferred from studies of silencing in yeasts and flies.

Heterochromatin can itself recruit its own inhibitors that limit its spread via reader-eraser coupling (**FIG. 3e**). An example is the Epe1 (enhancement of position effect) protein, a putative H3K9 demethylase recruited by *S. pombe* Swi6 [146-149]. Epe1 is degraded by a ubiquitin ligase that acts within the body of heterochromatin but not at its edges, providing a mechanism by which heterochromatin can recruit an anti-silencing factor whose activity is restricted[150]. Epe1 acts in parallel with boundary elements since loss of both Epe1 and TFIIIC sites that flank the mating type locus result in extensive heterochromatin spreading and slow cell growth[151]. Likewise, cells lacking both Epe1 and a globally-acting histone acetyltransferase (mst2; note H3K9ac prevents H3K9me) display widespread ectopic heterochromatin assembly and slow growth, again emphasizing the importance of redundant anti-silencing mechanisms[152]. Ectopic heterochromatin formation in such double mutants suggests that the processes which trigger heterochromatin at the main genomic locations act elsewhere, but are normally less effective. The detection of low levels of H3K9me at several loci in wild-type cells, under specific conditions, or in mutant backgrounds may be a manifestation of pathways important for gene regulation in response to various cues[93,152-156].

Tethering silencing machinery to its sites of prior action provides another mechanism to restrict heterochromatin to particular loci. Numerous chromatin-modifying enzyme complexes harbor domains that recognize the products of their respective reactions. In the budding yeast *Cryptococcus neoformans*, a H3K27-specific histone methyltransferase complex, PRC2, contains a chromo-domain subunit, Ccc1 (chromodomain-and-coiled-coil), that recognizes the H3K27me mark. H3K27me3 is selectively generated over subtelomeric

regions in this yeast[157]. Mutations that prevent the 'reader' domain from recognizing the reaction product cause ectopic H3K27 methylation at centromeres. This ectopic methylation requires H3K9me at *C. neoformans* centromeres, indicating that tethering of PRC2 to its sites of prior action via reader-writer coupling suppresses a latent attraction of PRC2 to H3K9-methylated domains, perhaps via the methyl-lysine binding activity of Eed.

6. Heterochromatin can be inherited

During replication, the H3-H4 tetramer subunit of old, parental nucleosomes are randomly distributed to nascent sister-chromatids during their synthesis (reviewed in[158]). New nucleosomes are assembled in the resulting gaps from free histones. The recruitment of KMTs by the modification that they catalyze ('reader-writer coupling') suggests that heterochromatin might self-propagate in a manner not dependent on the underlying DNA sequence (**FIG. 4a**). Such a property would enable information in the form of silent chromatin to be carried, along with any associated properties, through DNA replication into progeny cells. Such inheritance is termed '*cis* inheritance of a chromosomal state'. Similar to spreading mechanisms (discussed above), the recognition of H3K9me on parental nucleosomes by a reader-writer combination should allow the installation of that modification on these newly assembled neighboring nucleosomes.

Epigenetic inheritance is well known to be mediated by DNA methylation in some systems where a maintenance DNA methyltransferase (DNMT1) associated with the replisome recognizes 5meC in a CG dinucleotide and adds a methyl group to cytosine in the CG of the complementary strand (reviewed in [159]). In the filamentous fungus *Neurospora crassa*, H3K9 methylation and 5meC can reinforce each other; H3K9me nucleosomes can recruit the DIM-2 (defective-in-methylation) DNA methyltransferase via HP1 while DNA methylation recruits the H3K9 methyltransferase DIM-5[14,160]. In other systems, connections between H3K9me

and DNA methylation are also now well-established (reviewed in [161]). Because 5meC on CG dinucleotides is heritable through DNA replication, its influence on H3K9me could mask the *cis*-inheritance of chromatin states mediated by H3K9me read-write systems themselves.

Thus, a strong test of intrinsic H3K9me-marked heterochromatin heritability is persistence in a system lacking DNA methylation. DNA methylation is undetectable in fission yeast and stable *cis*-inheritance of heterochromatin occurs at the silent mating type locus[88,162]. Domains of synthetic heterochromatin form when the Clr4 SET-KMT domain is fused to an exogenous DNA binding domain and recruited to cognate binding sites placed at neutral euchromatic chromosomal loci, resulting in the silencing of embedded genes[163]. Use of a DNA binding domain controlled by a small molecule allowed conditional release of this artificial heterochromatin nucleator to test if endogenous wild-type Clr4-KMT, along with other effector proteins, could maintain heterochromatin and gene silencing through cell division (**FIG. 4b**)[164,165]. Release of tethered Clr4 resulted in rapid loss of H3K9me, even during a cell cycle block, suggesting that rather than being passively diluted through rounds of replication, H3K9me is actively removed. The histone demethylase Epe1 was found to be responsible for rapid ectopic H3K9 methylation removal. Cells lacking Epe1 can transmit H3K9me at the target locus through multiple cell divisions and even through meiosis into progeny. Thus H3K9 methylation has the potential to act as a heritable entity that also affects phenotype. Nonetheless, even in the absence of Epe1 anti-silencing activity, such engineered H3K9-methylated heterochromatin and associated gene silencing eventually dissipates, presumably due to imperfect copying during replication and/or transcription-coupled loss of H3K9me nucleosomes.

Analogous transient targeting experiments in mammalian cells suggest that H3K9me-mediated repression is reversible whereas DNA methylation allows the silent state to persist for many cell divisions without the trigger[166,167]. Thus, mammalian cells also appear to restrict the heritability of H3K9me-mediated repression after the initial recruiting mechanism is

disabled. In contrast, silent H3K9me-dependent heterochromatin formed by tethering HP1 persisted for many cell divisions following HP1 release from an engineered murine locus, although a potential role for DNA methylation in its maintenance at this locus seems difficult to rule out[168].

There is now strong evidence that the Polycomb silencing system, can mediate the *cis*-inheritance of a chromatin state[169]. Interestingly, in *Drosophila*, specific sequences that promote silencing machinery recruitment are required for this inheritance, suggesting again that the propensity of this type of silent chromatin to be inherited is tightly regulated, in this case positively by licensing a region for *cis*-inheritance[170,171]. Thus Polycomb silencing exhibits similarity to heterochromatin assembly at the *S. pombe* mating type locus which also involves sequence-specific binding proteins[162,172,173]. In the latter case, inheritance/maintenance is further promoted by chromatin remodeling enzymes which curbs nucleosome turnover, limits euchromatin assembly, and impacts positioning in the nucleus[174-176].

7. Heterochromatin mediates genome defense

Repetitive sequences are a threat to genome stability and organismal viability. Mechanisms of destabilization include the insertion mutations produced by transposable elements, DNA breaks produced by transposon excision, recombination between repeats, and replication stress and associated DNA breaks produced by repeats. Heterochromatin plays a pivotal role in suppressing these deleterious events through diverse mechanisms.

Studies in plants have revealed increased transposon copy number levels in mutants defective in the RNA-dependent DNA methylation (RdDM) pathway described above[177]. Surprisingly, only a single *copia*-type retrotransposon, EVD (evadé), increases when this pathway is mutated. Additional analyses confirm this observation[178], which has several

potential implications. It suggests that it is the latent activity of this single transposon that drives maintenance of RdDM in *Arabidopsis*. This might seem counterintuitive but theoretical work shows that a single element can spread through a sexually-reproducing population despite a negative impact on fitness[179]. The lack of impact of RdDM pathway loss on the copy number of other transposons, despite an increase in their transcript levels, suggests that these other elements might not be active or that other mechanisms limit their transposition. Their silencing could be important nonetheless for genetic stability as described below.

In *C. elegans*, loss of Piwi proteins (described above), that act upstream of a nuclear RNAi pathway coupled to H3K9me, has been shown to impact the movement of Tc3 transposons[101,180]. Recent studies of worms lacking H3K9-KMTs (*met-2* and *set-25*) detected widespread up-regulation of transposon transcripts in both germline and somatic tissues. Strikingly, this resulted in R-loop formation, replication stress and increased mutation frequency within repetitive elements[180]. Thus transcribed transposons can be mutagenic even without undergoing transposition *per se*.

Another less-appreciated aspect of heterochromatin is that it can control transposon activity by promoting specialized small RNA biogenesis mechanisms, rather than transcriptional silencing. In the Drosophila female gonad, mutations in the HP1 paralog Rhino result in defective piRNA biogenesis from clustered elements[181]. This is highly reminiscent of the role of H3K9me in siRNA biogenesis in *S. pombe*. piRNA clusters are heterochromatin islands that produce transposon-homologous small RNAs called piRNAs[182,183]. piRNAs act transcriptionally and post-transcriptionally to silence transposable elements[182]. Transposon insertion into a cluster is a mechanism by which the activity of that transposon is monitored and silenced in female gonads. The piRNA system also operates in mammalian testes to silence transposons via DNA methylation[184,185,186].

An important mechanism in genome defense is the avoidance of chromosomal rearrangements following DNA damage within repetitive elements. Homologous recombination (HR) between repeats (example: dispersed TEs) can result in deletions, inversions and translocations. *In cis* HR within repeated arrays, or with sister-chromatids, often results in expansion and contraction events that may cause little harm to cells and organisms (an exception being recombination within rDNA arrays[187]). In contrast, HR between repeats on non-homologous chromosomes can cause translocations and result in the formation of dicentric and acentric chromosomes. Studies in Drosophila and mammalian cells demonstrated that breaks within heterochromatin are sequestered to the periphery of heterochromatin compartments[188-190]. This is thought to promote repair by HR within the array or with sister-chromatids and thereby prevent illegitimate recombination with similar repeats on non-homologous chromosomes[191,192]. Heterochromatin may direct such events, limiting the repair of breaks to similar repetitive elements on sister-chromatids or the homologous chromosome, and thereby preventing deleterious rearrangements.

8. Heterochromatin influences centromere function

Centromeres are the chromosomal loci where kinetochores assemble. Most eukaryotic centromeres are composed of repetitive DNA arrays; the majority of these repeats are embedded in H3K9me-heterochromatin and are heavily 5meC/DNA methylated in mammalian somatic cells. However, patches of repeats assemble unusual nucleosomes in which histone H3 is replaced by a variant, CENP-A (centromere protein A). These centromere-specific nucleosomes form the physical foundation for the kinetochore (reviewed in [193]). Heterochromatin plays several important roles.

First, heterochromatin influences the assembly of CENP-A chromatin domains. In *S. pombe*, CENP-A chromatin and functional kinetochores cannot be established on transformed

centromere DNA lacking flanking pericentromeric heterochromatin. Heterochromatin provides a critical, but unknown, function to ensure CENP-A chromatin assembly on adjacent sequences. Heterochromatin-directed histone modifications and/or nuclear periphery association may promote CENP-A incorporation. Heterochromatin may also act to limit the size of the CENP-A/kinetochore domain[131,194]. Conversely, inadvertent or forced heterochromatin formation within fission yeast[195] or mammalian cell centromeres prevents CENP-A and kinetochore assembly[196,197].

A second role for heterochromatin at centromeres involves sister-chromatin cohesion, mediated by cohesin[198]. At metaphase, most metazoan sister-chromatids remain associated via cohesion only at their centromeres. This is because centromeric cohesin, that embraces both sisters, is protected from degradation until anaphase. In *S. pombe*, centromeric heterochromatin is required to mediate tight physical sister-centromere cohesion by trapping high levels of centromeric cohesin. This occurs through physical association of the cohesin complex with heterochromatin via Swi6[HP1] [199,200]. In cells lacking heterochromatin, single kinetochores are disorganized and display aberrant attachment to spindles. Sister-centromeres also prematurely dissociate, leading to chromosome loss and gain[201-203]. This explains the frequent chromosome loss events observed in *S. pombe* cells with defective heterochromatin[8,203]. Sister-centromere cohesion may also be weaker in human cells exhibiting reduced levels of centromeric H3K9me heterochromatin. Such a defect was reported for human cancer cell lines exhibiting chromosome instability due to overexpression of KDM4 H3K9me3 demethylases[204].

9. Heterochromatin controls differentiation

Evolution has also put heterochromatin to work to accomplish additional functions. An example in *S. pombe* is the silencing of gene cassettes that each encode two transcription factors that program cell type. The heterochromatin domain that silences these cassettes is

called the mating-type *mat2-mat3* region. A lineage-regulated recombination event places copies of these transcription factor encoding genes into the expression site (*mat1*) producing a change in mating-type[205,206]. In addition to silencing *mat2-mat3*, H3K9me heterochromatin plays a role in regulating the directionality of this recombination and therefore the pattern of mating-type switching[206].

Megabased-sized islands of H3K9me-dependent heterochromatin are formed in a mammalian cell type-specific manner[207]. One function of these islands is to form a barrier to transcription factor-mediated cell-type reprogramming; hence they are termed differentially bound or reprogramming resistant regions (**FIG. 5a**). This H3K9me-dependent heterochromatin is important for preservation of differentiated cell type identity since depletion of proteins involved in maintenance of this heterochromatin (CAF, chromatin-assembly-factor; SETDB1, SET-domain-bifurcated 1; KAP-1^{TRIM28}, KRAB-A-interacting-protein) allows more efficient reprogramming of differentiated cells to iPS cells[207-211] or of somatic nuclei transferred to oocytes[212]. The *cis*-determinants required to establish these large heterochromatin islands remain unknown, but initiation may be linked to mechanisms that silence endogenous retroelements (EREs, including ERVs) in somatic cells (**FIG. 5b**). ERE reactivation can result in inappropriate expression of neighboring genes. The silencing mechanisms used to inactivate EREs are related to those that block reprogramming of somatic cells. A family of KRAB-ZFP (Kruppel-Associated-Box-Zinc-Finger) proteins are known to recruit SETDB1 H3K9 methyltransferase via the adaptor KAP-1^{TRIM28} to EREs where they elicit repressive heterochromatin[213-216]. Thus, ancient transposable elements appear to have been co-opted for regulation of adjacent chromatin landscape and nearby genes.

10: Heterochromatin is medically important

Heterochromatin plays roles in human disease. We focus here on a handful of examples among many that illustrate and extend important principles.

Viral latency: Heterochromatin protects genomes from pathogenic viruses. For example, a fraction of Type 1 Human Immunodeficiency Virus (HIV-1) integrations can occur within heterochromatin regions[217]. Retroviral reporters in lymphocyte cell lines are subject to silencing by H3K9me-mediated heterochromatin via the HUSH (Human Silencing Hub) Complex which spreads over the viral genome from neighbouring heterochromatin[218] (**FIG. 5c**). Although speculative, silencing of integrated viruses may allow dormant HIV-1 retrovirus to persist in AIDS patients' T cells, long after therapeutic clearance of circulating virus. Sporadic reactivation of these proviruses may enable later reappearance of viruses. Interestingly, in this case a distinct chromo-domain protein (MPP8, M-phase phosphoprotein 8, not HP1) binds H3K9me3 directed by SETDB1 KMTase. Other human viruses may also be rendered dormant by HUSH-mediated heterochromatin speading[217]. HUSH silencing is distinct from that mediated by KRAB-ZFPs which target heterochromatin formation to retroviruses and EREs (see above)[213-217,219].

Obesity: The increasing frequency of obesity in humans, and associated health risks, has a heritable component. Intriguingly, KAP-1^{TRIM28} (a major heterochromatin recruitment platform) haplo-insufficiency in mice results in stochastic production of either normal or obese offspring from genetically identical parents. Analyses of human lean and obese cohorts indicates that KAP-1^{TRIM28} expression levels correlate with expression patterns of key obesity-associated genes and body mass index[220].

Premature aging: WRN gene (encodes a helicase) mutations cause Werner Syndrome, an adult form of progeria (premature aging). WRN null human mesenchymal stem cells (MSCs) display disrupted heterochromatin with loss of H3K9me3 from heterochromatin islands[221]. The WRN protein is targeted to centromeric repeats and associates with Suv39H1 H3K9

methyltransferase and HP1α. This WRN complex may stabilize repeat arrays within heterochromatin, preventing DNA damage. Comparison of primary human MSCs from young and old individuals revealed reduced levels of WRN protein and heterochromatin loss in old cells. The implication is that WRN protects heterochromatin and thereby prevents the irreversible genome instability associated with aging. Alternatively, it could be that DNA damage induces loss of heterochromatin.

Metabolism: DNA and histone methyltransferases and demethylases require metabolites for their activities (reviewed [222,223]) S-adenosylmethionine is the methyl donor for nucleic acid and histone methyltransferases. Many demethylases require α-ketoglutarate, a metabolic intermediate of the Krebs cycle, as a co-substrate, while others utilize flavin adenine dinucleotide. Moreover, acetyl-CoA is the acetyl donor for histone acetyltransferases and the sirtuin family of histone deacetylases requires nicotinamide adenine dinucleotide as a cofactor. Consequently, changes in the nutritional environment or mutations that affect levels of metabolites can cause the accumulation of inhibitory products, which can alter chromatin.

For example, mutations in the genes encoding isocitrate dehydrogenase, fumarate hydratase and succinate dehydrogenase, key Krebs cycle enzymes, cause accumulation of the substrates 2-hydroxyglutarate, fumarate and succinate, respectively, which are competitive inhibitors of α-ketoglutarate-dependent histone and DNA demethylases[224,225]. Such mutations promote tumors. Accumulation of 2-hydroxyglutarate results in elevated H3K9me levels and blocks cellular differentiation[224]. Conversely, provision of α-ketoglutarate to ES cells reduces histone and DNA methylation and promotes pluripotency whereas succinate has the opposite effect. Histone methylation in ES cells is sensitive to glutamate, and thus α-ketoglutarate, supply[226]. Poor nutrient availability is a feature of many solid tumors, where interior regions are deprived of glutamine, and hence α-ketoglutarate, leading to elevated histone methylation and cellular dedifferentiation within such tumors[227]. In *S.*

cerevisiae, equivalent mutations to those that cause 2-hydroxyglutarate accumulation were found to enhance SIR-mediated silencing by inhibiting H3K36 methyltransferases[228].

Concluding remarks:

We have described general principles that have emerged from the study of heterochromatin in a broad range of organisms and have provided a few case studies to illustrate each. Among many unanswered questions in the field, several stand out: What are the signals that initially trigger heterochromatin at specific sites? What determines the heritability or lack or heritability of heterochromatin? What is the role of phase separation in heterochromatin integrity? What enables transcription of heterochromatin? How is heterochromatin regulated during stress and development? We anticipate that model organisms, new technologies and ingenious experimental strategies will be required to address these outstanding issues.

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None

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BOX 1 : Heterochromatin history

Heterochromatin was first used as a cytological term coined by Emil Heitz in 1928 who developed staining methods that revealed regions of chromosomes with distinct behaviour. He found that chromosomes stained differently and consistently along their lengths. He called those regions not visible after telophase 'euchromatin' and those remaining discernible (heteropyknotic) 'heterochromatin'. He noted that staining patterns are specific for each chromosome and later related these to the genetic properties of chromosomes, suggesting that genes are found in euchromatin whereas heterochromatin is genetically inert. He also noted that heterochromatin is often associated with sex chromosomes [reviewed [1]]. Finally, he recognized that there were regions that sometimes appear distinct, later termed facultative heterochromatin, and those that are always distinct, latter dubbed constitutive heterochromatin.

In the early 1930s, after exposing Drosophila to X-rays, Hermann Muller isolated the *white mottled* mutations that exhibited a mosaic or variegated pattern of red (wild-type) or white (mutant) facets of the eye due to chromosome rearrangements that displaced the *white* gene from its original position (hence his term 'eversporting displacements' in that each individual developed a different eye color pattern)[229]. In 1936, the examination of polytene chromosomes revealed that rearrangement breakpoints within heterochromatic regions were frequently associated with such variegating mutants[230]. Thus, the vague cytological entity 'heterochromatin' became intertwined with a phenomenon that was dubbed position-effect variegation (PEV; variegation in a phenotype due to the variable inactivation of a gene triggered by its placement in or near heterochromatin). Extra copies of heterochromatic chromosomes were found to alleviate this PEV, perhaps because they titrated limiting factors[231,232]. Later, mutations were isolated in single genes that increased or decreased the variegated eye color phenotype[233-235].

Exploration of genomes in the 1960s using re-association kinetics of sheared denatured DNA revealed that a significant fraction of eukaryotic genomes are repetitive[236]. These rapidly annealing fractions were found to correspond to components of genomes that exhibit a distinct buoyant density on CsCl gradients due to their skewed base composition relative to the rest of the genome[237,238]. Because they formed a peak in the density profile that was not coincident with the bulk of the genome, the sequences within these ancillary peaks were termed 'satelllites.' Since satellite peaks form with both sheared, low-molecular weight and high-molecular weight DNA it was concluded that the constituent repeats occur in arrays[239-241]. The collapse of satellite DNAs to homogeneous repeat-length bands by digestion with restriction enzymes that cut once per repeat confirmed that such satellite repeats are arranged in long tandem arrays. Because of their abundance, satellite DNAs were the first eukaryotic DNAs to be sequenced by early methods[242,243].

The use of purified satellite DNAs as labelled probes for *in situ* hybridization to metaphase chromosomes revealed that these DNAs are located in the centromeric heterochromatin regions of metaphase chromosomes[244,245] and co-localize with dense chromatin at the nuclear periphery during interphase[246]. Thus, it became apparent that large blocks of constitutive pericentromeric heterochromatin contain arrays of repetitive satellite DNAs and that artificial juxtaposition of genes with such regions by a chromosomal rearrangement led to their inactivation.

The above findings coupled with the inability to detect RNA complementary to satellite suggested that they are transcriptionally inactive domains with no genetic output[247]. Moreover differential centrifugation showed that the sedimentation characteristics of satellite DNA heterochromatin were consistent with this chromatin being more compact[248]. These regions were also found to be late replicating[249] and under-replicated in polytene nuclei[250] suggesting that heterochromatin might also affect DNA replication.

BOX 2 : On the terms 'epigenetic' and 'epigenetics'

Waddington originally coined the term 'epigenetics' to refer to the mechanisms that mediate acquisition of stable cell fates during development, but many individuals subsequently modified its definition (reviewed in [251]). The term epigenetics was altered by Holliday to refer to the inheritance of changes in gene expression patterns and, more generally, the inheritance of any change in gene function that does not involve a change in DNA sequence. Riggs defined epigenetics as 'the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence'. Ptashne defined the phrase 'epigenetic change' as a heritable change in the expression of a gene that does not involve a change in its sequence and persists in the absence of the initiating signal. Bird questioned whether heritability should be a compulsory component of a modern epigenetics definition because it does not specify how many generations of inheritance might be required to satisfy the definition. He suggested 'the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states' as an all-encompassing definition[252]. This chromosome-based definition excludes any number of other feedback mechanisms that can mediate heritable change without a change in DNA sequence such as post-transcriptional positive feedback loops (as occurs in Drosophila sex determination and in prions).

Despite these foundational definitions, the use of the noun 'epigenetics' and the adjective 'epigenetic' has been essentially redefined by many to refer to chemical modifications of histones and DNA because, in some cases, these are required for/contribute to a heritable change in gene expression. The adjective "epigenetic" has thus been used in the context of phrases such as "epigenetic mark" or "epigenetic modification" in a manner synonymous with chemical modification of nucleic acid or associated protein (more generally a 'chromatin modification'). The ensemble of such modifications has been referred to as the 'epigenome.' Such extensions, while entrenched, may be misconstrued or imply an untruth (depending on

the definition being applied), namely that any chemical modification of a nucleic acid or associated protein mediates a heritable change in the expression of a gene.

FIGURE LEGENDS

Figure 1. Core heterochromatin components and mechanisms

a | Depiction of a heterochromatin protein 1 (HP1) dimer bound to nucleosomes methylated on lysine 9 of their N-terminal H3 tails (H3K9me; red circles). The chromo-domain (CD; orange crescent) and the chromoshadow domain (CSD; green object), a dimerization domain, of HP1 are shown. The platform produced by the CSD dimer enables binding of effector proteins (yellow object). For simplicity, only one of the two H3 tails (black wave) that protrude from the octamer core is shown for each nucleosome (grey ovals). **b** | Clr4 and Suv39 histone H3 lysine 9 methyltransferases (KMTs). The protein domain structures of Clr4 (Cryptic-loci-regulator-4; *S. pombe*), SU(VAR)3-9 (Suppressor-of-variegation-39; *D. melanogaster*) and Suv39H1 (homolog 1; *H. sapiens*). The chromo-domains (CD) of these proteins specifically recognize the H3K9 methylated tail, the product of the reaction catalyzed by these KMTs. The SET domain is the catalytic domain and uses S-adenosylmethionine as a methyl-donor. **c** | Heterochromatin assembly and disassembly by reader-modifier coupling. In this generalized scheme, different enzymes catalyze the addition ("writers"; blue oval) of a post-translational modification (PTM; red circle) to a histone within a nucleosome, or its removal ("erasers"; black pac-man). The PTM results in the direct recruitment of proteins ("readers"; orange crescent). Writer or eraser modules are often coupled with reader modules, either residing in the same polypeptide (as in b), or protein complex, or via reversible protein-protein interactions. **d** | Recruitment mechanisms. In some systems, DNA binding proteins (DBP; green objects) have been identified that recruit writers (blue oval) or erasers (black pac-man). In other systems, a nascent transcript (blue line) associated with template bound RNA Polymerase (grey object) provides a recognition platform. This RNA harbors signals for a sequence-specific and/or structure-specific ribonucleoprotein (RNP; purple object), or RNA binding protein (RBP; brown object). The latter include Argonaute family proteins that recognize and bind cognate RNA via incorporated small RNAs (e.g. siRNA, piRNA; reviewed [253]). The RBP/RNP in turn can recruit writers or erasers that modify chromatin.

Figure 2. Determining if a factor is required for establishment, but not maintenance, of heterochromatin

Identifying a factor that is required to maintain repressive heterochromatin is straightforward since deletion of the gene encoding that factor will disrupt heterochromatin formation and associated phenotypes such as silencing. Determining whether a factor has a role in its establishment requires additional steps. **a** | The gene for an endogenous pivotal writer is inactivated resulting in the loss of a heterochromatin domain (red rectangle) such as that mediated by H3K9me (red circles) in these wild-type cells. A heterochromatin associated entity is represented by "X" (yellow X; a protein, an RNA or a PTM). **b** | Restoration of the writer to otherwise wild-type cells allows re-establishment of a full heterochromatin domain indicating that all factors required for its nucleation, spreading and maintenance are present including X. **c** | Cells lacking the heterochromatin associated "X" factor are utilized in the same test. Note: X may be required for establishment but not strictly required for maintenance. **d** | The full assembly of a silent heterochromatin domain upon resupply of the writer indicates that X is not required for nucleating heterochromatin formation. **e** | The inability to re-establish a full heterochromatin domain indicates that X is required for upstream events that trigger heterochromatin assembly, but is not required for its maintenance. RNAi in *S. pombe* and XIST RNA in mammals are examples of such establishment factors.

Figure 3. Heterochromatin spreading and mechanisms that restrict expansion

a | A model for the expansion of a heterochromatin domain in which a "reader" module (orange crescent) is associated with a "writer" module (blue oval), thereby causing the PTM (red circle) on one nucleosome (grey oval) to enhance the rate of modification on a nearby nucleosome. Iterative cycles result in the formation of extensive heterochromatin domains bound by many factors recruited by the initiating PTM. A barrier (dashed curved grey lines) represents a series of mechanisms that restrict such spreading, shown on the right. **b** | Sequence bound factors (striped rectangle) that disfavor nucleosome assembly create extensive gaps, or topological entities, which prevent heterochromatin from spreading. **c** | factors that promote nucleosome turnover through disassembly-reassembly and/or subunit exchange cycles (light ovals with arrows) effectively break the ability of heterochromatin domains to expand. **d** | Adjacent expressed transcription units mediate the addition of active PTMs (green circles) to histones which prevent the intrusion of repressive H3K9me-dependent heterochromatin. **e** | Eraser modules (back pac-man) that are strategically recruited at the edge of heterochromatin regions can remove the key PTM (white circles, red outline) and prevent expansion. (e.g. *S. pombe* Enhancer-of-position-effect, Epe1 demethylase counters H3K9me).

Figure 4. Reader-writer coupling allows repressive chromatin modifications to be copied during replication and transmitted through cell division

a | A model for the maintenance of a repressive PTM through DNA replication by reader-writer coupling. During replication H3-H4 tetramers from preexisting parental "old" nucleosomes (dark ovals) are randomly recycled to either of the two nascent strands. Consequently, the number of H3 nucleosomes bearing a PTM, such as H3K9me, on the two new strands will be reduced by half compared to the parental domain. Reader (orange crescents) – writer (blue ovals) coupling allows copying of the PTM from "old" nucleosomes that retain the PTM to newly assembled nucleosomes (light ovals) thereby replenishing and reinstating the full chromatin domain on both sister-chromatids and ultimately allowing its transmission to progeny cells. **b** | A writer module (W; blue oval), such as the SET domain of an H3K9 methyltransferase, is artificially recruited by fusion to a DNA-binding domain (DBD; light blue object) whose binding site (light blue diamond) is inserted at a neutral genomic location. This generates a region harboring a specific chromatin PTM, such as H3K9 methylation (red circles on tails of nucleosomes) which can recruit additional reader-writers (orange crescent) that recognize that PTM and can spread the PTM over a nearby reporter gene, silencing its expression (yellow rectangle). Release of the triggering artificial writer from DNA by inhibition of its DNA-binding domain allows persistence and heritability of this chromatin to be assessed. **c** | If transcriptional repression and gene silencing are maintained through cell division (by replication fork associated reader-writer coupled copying as in **a**), then the modification, in this case H3K9me, must mediate a heritable epigenetic change [Box 2].

Figure 5. Heterochromatin domains, transposable elements and reprogramming resistance in mammalian cells

a | The forced expression of four transcription factors (Oct4, Sox2, Klf4 and c-Myc; OSKM) induces dedifferentiation of somatic cells (orange star shapes) of various lineages to an induced pluripotent state (iPS cells; blue ovoid shapes). Such reprogramming is inefficient because large heterochromatin domains (depicted by red rectangle) present a barrier to the activation of key genes required for reversion to pluripotency. Increased reprogramming efficiency can be achieved by depletion proteins such as KAP-1^{Trim28} and SETDB1, which are required for heterochromatin integrity, allowing activation (green dashed rectangle) of reprogramming pathways. **b** | Full length and fragments of mammalian transposable elements including Endogeneous Retro-Elements (EREs) are bound by members of the large KRAB-ZFP (KZFP; Kruppel-Associated-Box-Zinc-Finger turquoise rectangle) family of proteins and act as nucleators, mediating H3Kme heterochromatin formation (red circles) by recruitment of the H3K9me 'writer' methyltransferase SETDB1 (light blue rectangle) via the adaptor protein KAP-1^{Trim28} (yellow rectangle). This in turn allows recruitment of H3K9me 'readers' (such as HP1 and MPP8) and writers to expand the domain. Spreading of heterochromatin outwards can silence adjacent genes suggesting that TE remnants have been co-opted for host (hetero)-chromatin domain regulation. **c** | Reporter constructs occasionally insert in heterochromatin islands on chromosome arms where the HUSH complex (MPP8, Periphilin PHL, TASOR, SETDB1) spreads heterochromatin into, and silences, the reporter. The 'reader' MPP8 (orange crescent) binds flanking H3K9me (red circles) and recruits the H3K9me 'writer' SETDB1 (blue oval) via the adaptor protein TASOR (yellow object). This silencing mechanism may be used to render pathogenic viruses latent. HUSH might also promote heterochromatin island formation by mediating spreading from TEs or EREs.

Glossary *(underlined at first mention in text)*:

Satellite repeats: repetitive components of genomes (generally tandem arrays of short elements) that exhibit a distinct satellite peak on buoyant density gradients due to their skewed base composition relative to most genomic DNA.

Post-translational modification PTM: Chemical groups such as Methyl (me, $-CH_3$), that are enzymatically added to (by 'writers'), or removed from (by 'erasers'), amino acids side chains of proteins and are bound by particular protein modules ('readers').

Constitutive heterochromatin: heterochromatin that is consistently formed though the cell cycle and in many cell types in most eukaryotes (example centromere associated heterochromatin).

SET domain: conserved protein modules that exhibit methyltransferase activity which adds methyl groups to the ε-amine groups of lysine residues in histones (example: Suv39).

chromo-domain CD: conserved protein module (example: HP1-related proteins) that can bind methylated lysine residues on histones such as H3K9me, H3K27me.

chromoshadow domain CSD: dimerization domain within HP1-related proteins that forms a peptide-binding groove at the dimer interface that can recruit additional heterochromatin proteins.

facultative heterochromatin: locus and cell type specific heterochromatin (example inactive X chromosome)

Argonaute: PAZ and PIWI domain proteins that are loaded with small RNAs which guide them, and associated proteins, to long RNAs bearing homology to the small RNA.

X chromosome inactivation: the process of dosage compensation in female mammals where one of the two X chromosome is inactivated by facultative heterochromatin formation.

XIST: long non-coding RNA that designates the copy of the X chromosome from which it is expressed for silencing in mammals.

piRNAs: small RNAs associated with Piwi members of Argonaute protein superfamily, which promote transposable element repression in animal gonadal tissues.

copia-type retrotransposon: a widespread transposable element family that mediates their own replication and insertion at new sites in genomes.

R-loops: nascent RNA that remains associated with its template through hybridization thereby dislodging the opposite non-templating DNA strand.

Heterochromatin islands: extensive domains of heterochromatin on chromosome arms that are distinct from the main centromeric and telomeric heterochromatin domains.

Pericentromeric heterochromatin: large blocks of heterochromatin formed on tandem repeat arrays that surround the centromere-kinetochore region.

Reprogramming resistant regions: similar to differentially bound regions, large lineage specific chromosomal regions assembled in heterochromatin and resistant to reprogramming factor binding.

Endogenous retroelements ERE: full length mobile elements that replicate and insert elsewhere in genomes; also includes immobile degenerate ERE fragments.

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Figure 1

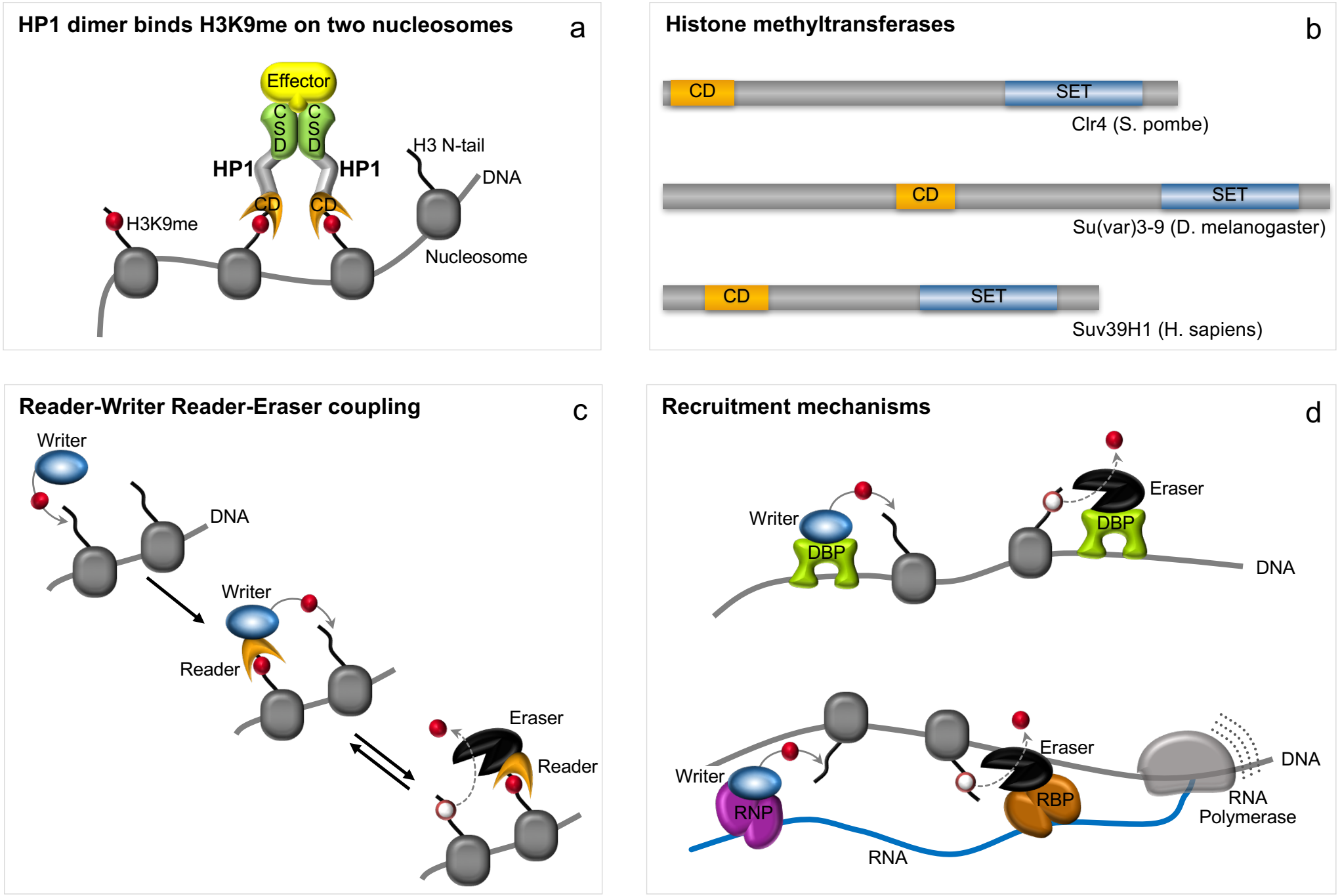

HP1 dimer binds H3K9me on two nucleosomes
a
Effector
CSD
CSD
HP1
HP1
CD
CD
H3 N-tail
DNA
H3K9me
Nucleosome
Histone methyltransferases
b
CD
SET
Clr4 (S. pombe)
CD
SET
Su(var)3-9 (D. melanogaster)
CD
SET
Suv39H1 (H. sapiens)
Reader-Writer Reader-Eraser coupling
c
Writer
DNA
Writer
Reader
Eraser
Reader
Recruitment mechanisms
d
Writer
DBP
Eraser
DBP
DNA
Writer
RNP
Eraser
RBP
DNA
RNA
Polymerase
RNA

Figure 2

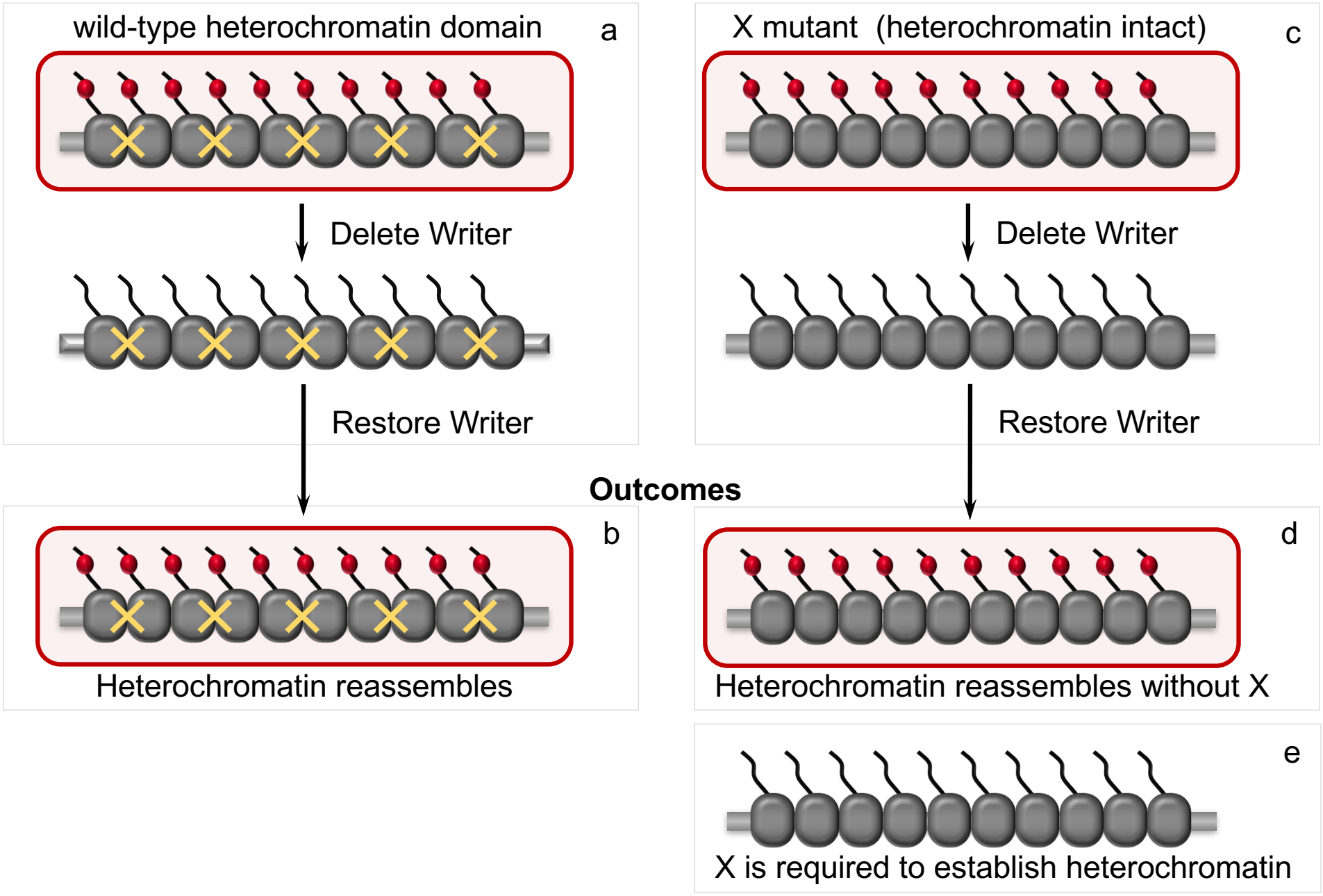

wild-type heterochromatin domain
a
Delete Writer
Restore Writer
b
Heterochromatin reassembles
X mutant (heterochromatin intact)
c
Delete Writer
Restore Writer
Outcomes
d
Heterochromatin reassembles without X
e
X is required to establish heterochromatin

Figure 3

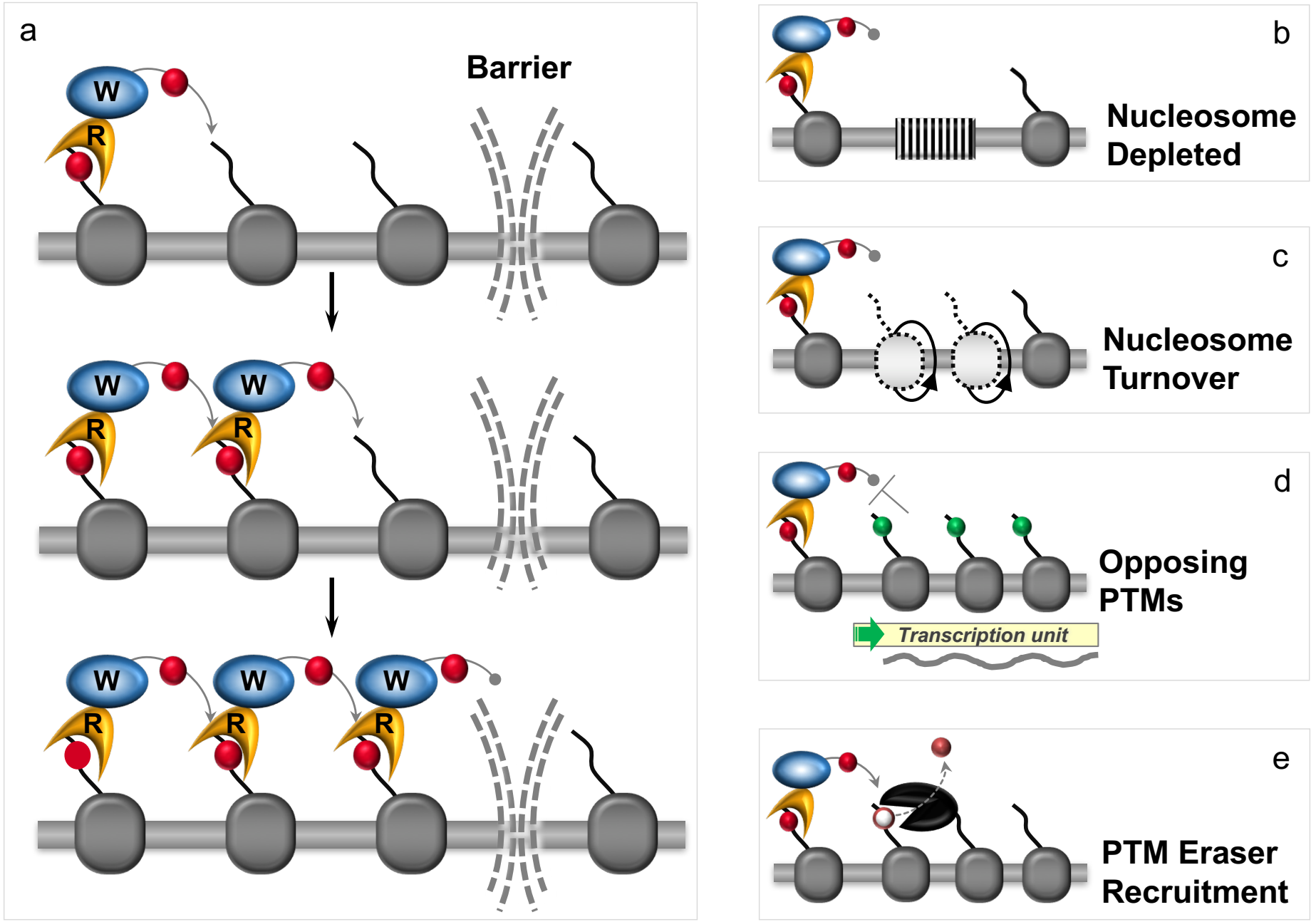

a
Barrier
W
R
b
Nucleosome
Depleted
c
Nucleosome
Turnover
d
Opposing
PTMs
Transcription unit
e
PTM Eraser
Recruitment

Figure 4

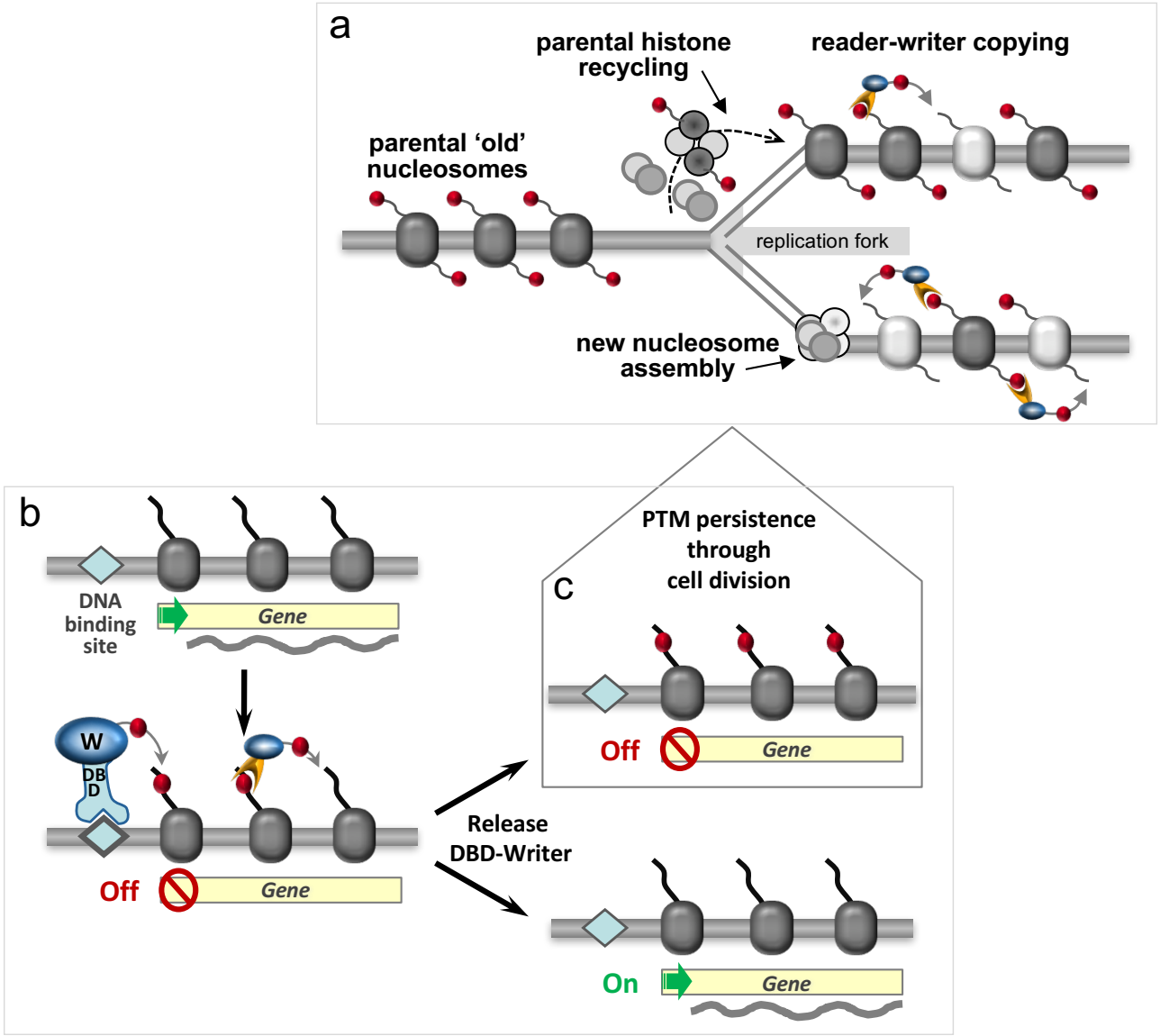
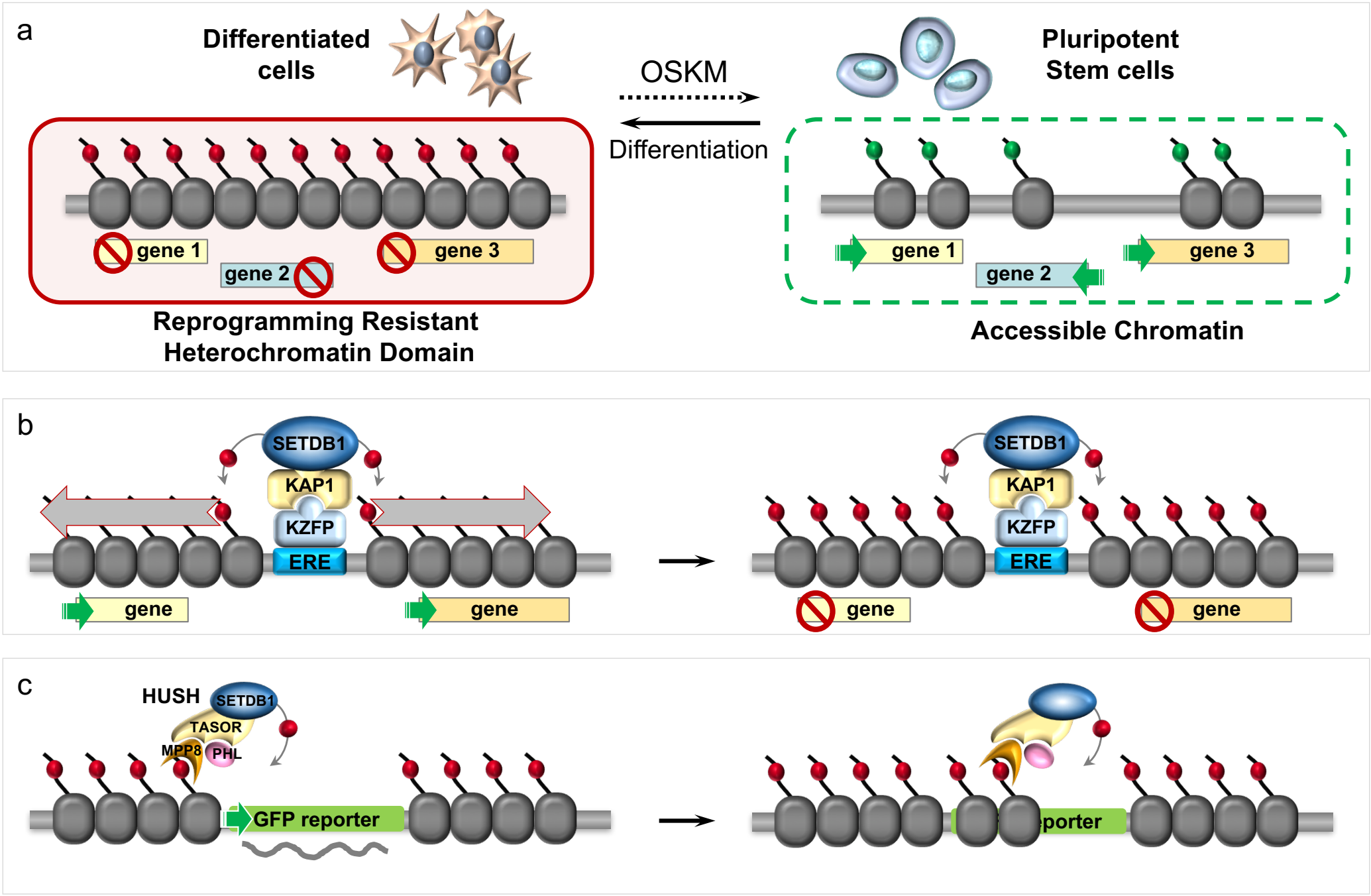

a
parental histone recycling
reader-writer copying
parental 'old' nucleosomes
replication fork
new nucleosome assembly
b
DNA binding site
Gene
W
DB D
Off
Gene
Release DBD-Writer
c
PTM persistence through cell division
Off
Gene
On
Gene

Figure 5


a
Differentiated cells
OSKM
Differentiation
Pluripotent Stem cells
gene 1
gene 2
gene 3
Reprogramming Resistant Heterochromatin Domain
gene 1
gene 2
gene 3
Accessible Chromatin
b
SETDB1
KAP1
KZFP
ERE
gene
gene
SETDB1
KAP1
KZFP
ERE
gene
gene
c
HUSH
SETDB1
TASOR
MPP8
PHL
GFP reporter
porter